Remarks

Claims 1, 6, 7, 10, and 14-21 are pending. Applicants appreciate the withdrawal of the rejection of claims 1, 6, 7, 10, and 14-21 under 35 U.S.C. § 112 first paragraph, written description and enablement.

Rejection Under 35 U.S.C. § 112, written description

Claims 1, 6, 7, 10, and 14-21 were rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention. Applicants respectfully traverse this rejection.

The claims have been amended to recite that the methods include providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA. Support for this amendment can be found on page 4, lines, 3-8 and page 21, lines 11-15, which disclose that 3-hydroxyhexanoyl CoA accepting PHA polymerase genes can be obtained from *A. caviae, C. testosteroni, T. pfenigii,* and possibly *P. denitrificans* and *S. natans*.

Further support for the polyhydroxyalkanoate polymerase gene from *A. caviae* can be found in Example 2, on page 21, lines 24-26, which cites a publication (Fukui & Doi. *J. Bacteriol.* 179:4821-4830 (1997)) that describes the sequence for this gene. In addition, the Examples disclose that the polymerase from *A. caviae* (Examples 2 and 5) and the polymerase from *N. salmonicolor* (Example 3) can both form PHBH copolymers. This demonstrates that enzymes that accept both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA as substrates are

not mere conjecture, but substantiated. Finally, Figure 9 is a schematic of selection for a PHBH recombinant pathway in *E. Coli* using the PHA polymerase gene from *P. putida*. The schematic clearly shows that the polymerase acts on 3-hydroxyhexanoyl-CoA. Accordingly, it is clear from the ample support in the specification that the Applicants were in possession of polyhydroxyalkanoate polymerases with the appropriate substrate specificity.

Rejection Under 35 U.S.C. § 102

Claims 1, 7, 10, 15, 18, 19 and 20 were rejected under 35 U.S.C. § 102(b) as being anticipated by Timm A. et al. *Appl. and Environ.l Microbiol.* 56(11): 3360-3367 (1990) ("Timm") in light of Hoffman N. et al. *FEMS Microbiol. Lett.* 184: 253-259 (2000) ("Hoffman"). Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

The Legal Standard

For a rejection of claims to be properly founded under 35 U.S.C. § 102, it must be established that a prior art reference discloses each and every element of the claims. *Hybritech Inc v Monoclonal Antibodies Inc*, 231 USPQ 81 (Fed. Cir. 1986), *cert. denied*, 480 US 947 (1987); *Scripps Clinic & Research Found v Genentech Inc*, 18 USPQ2d 1001 (Fed. Cir. 1991). The Federal Circuit held in *Scripps*, 18 USPQ2d at 1010:

> Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. . . *There must be no difference* between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. (Emphasis added)

A reference that fails to disclose even one limitation will not be found to anticipate, even if the missing limitation could be discoverable through further experimentation. As the Federal Circuit held in *Scripps, Id.*:

> [A] finding of anticipation requires that all aspects of the claimed invention were already described in a single reference: a finding that is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations. The role of extrinsic evidence is to educate the decision-maker to what the reference meant to persons of ordinary skill in the field of the invention, not to fill in the gaps in the reference.

Analysis

The claims, as amended, specify that the method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate includes providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA.

Timm discloses transforming *Pseudomonas aeruginosa* with a polymerase (*phbC*) gene from *A. eutrophus* (*R. eutropha*). However, the first paragraph of the Discussion of Timm teaches "...the PHA synthase of *A. eutrophus* exhibits a preference for CoA derivatives of hydroxyalkanoic acids with four and five carbon atoms and **does not accept** longer derivatives as substrates", ***thereby specifically excluding the claimed subject matter***. The specification also teaches that the polymerase from *R. eutropha* does not polymerize 3-hydroxyhexanoyl-CoA. See page 11, lines 5-9, which reads "The PHB polymerase from *R. eutropha* is highly specific

for the 3-hydroxybutyryl CoA monomer and shows only 7.5% activity towards 3-hydroxyvaleryl CoA. No activity with **3-hydroxyhexanoyl CoA** or longer 3-hydroxyacyl CoA's was detected in *in vitro* studies (Haywood, et al., *FEMS Microbiol. Lett.*, 57:1 (1989)."

In addition, Table 1 of Timm shows that the control strain *P. aeruginosa* PAC1 synthesizes PHA containing 3-hydroxyhexanoate ***before*** the PHB-synthetic genes of *A. eutophus* are introduced (*P. aeuruginosa* PAC1 (pVK101::PP1)- see page 3361, Results Section, lines 2-4). Therefore, the ability of this strain to produce PHA containing 3-hydroxyhexanoate is due to endogenous enzymes expressed by the strain and not from the expression of the *A. eutrophus* genes.

Hoffman does not make up for this deficiency. Hoffmann was cited to prove that *Pseudomonas aeruginosa* inherently comprises the *phaG* gene, i.e. a gene encoding a Beta-hydroxyacyl-ACP-coenzyme A transferase also known as ACP-CoA acyltransacylase. Hoffmann states that the phaG gene product from P. aeruginosa is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain length constituents from non-related carbon sources. specifically Hoffmann states that phaG exhibits 3-hydroxyacyl-ACP:CoA transacylase activity

Since the disclosure of Timm in light of Hoffman does not teach providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA, the reference does not disclose all of the claim elements and limitations and, thus, does not anticipate the claims.

Rejection Under 35 U.S.C. § 103

Claims 1 and 6 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Timm, in view of U.S. Patent No. 5,470,727 to Macharenas et al ("Macharenas"). Claims 1, 7 and 14-21 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Timm, in view of Schubert P. et al. *J. Bacteriol.* 170(12): 5837-5847 (1988) ("Schubert") and in further view of Boynton Z. et al. *J. Bacteriol.* 178(11): 3015-3024 (1996) ("Boynton"). Applicants respectfully traverse these rejections to the extent that they are applied to the claims as amended.

The Legal Standard

U.S. Patent and Trademark Office has the burden under 35 U.S.C. § 103 to establish a *prima facie* case of obviousness. *In re Warner et al.*, 379 F.2d 1011, 154 U.S.P.Q. 173, 177 (C.C.P.A. 1967), *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596, 1598-99 (Fed. Cir. 1988). To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *In re Geiger*, 815 F.2d 686, 2

U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703, 705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

Analysis

Rejection of claims 1 and 6 over Timm, in view of Macharenas

As stated above, Timm discloses transforming *Pseudomonas aeruginosa* with a gene encoding PHB polymerase from *A. eutrophus* (*R. eutropha*), which does not polymerize 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Macharenas merely teaches the chromosomal expression of non-bacterial genes in bacterial cells.

Neither Timm nor Macharenas, alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Therefore, the claims of the present invention are not *prima facie* obvious over these references, since the references do not teach or suggest all of the claim limitations.

<u>Rejection of claims 1, 7 and 14-21 were over Timm, in view of Schubert and in further view of Boynton</u>

Both Timm and Schubert disclose introducing the PHB synthetic genes from *A. eutrophus* into *P. aeruginosa* and *E. coli*, respectively. The PHB polymerase from *A. eutrophus* does not polymerize 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. In addition, Schubert does not describe a polymerase gene from *R. rubrum* that was available. Schubert cites to a paper that was published in 1960 (Merrick, J.M. and Doudoroff, M. *Nature* 189: 890-892 (1960) (enclosed), which only states that PHB synthase is associated with phospholipids on the surface of the PHB granules under certain conditions of growth. There is nothing in the reference about isolating, cloning, sequencing or genetically engineering a PHB synthase from *R. rubrum*.

Boynton discloses three enzymes from *C. acetobutylicum* that form butyryl CoA.

None of these references, either alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Therefore, the claims are not *prima facie* obvious over these references, since the references do not teach or suggest all of the claim limitations.

Double Patenting Rejection

Claims 1, 6, 10 and 16 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 17 of U.S. Patent No. 6,593,116 and claim 18 of U.S. Patent No. 6,913,911. Claims 1, 6, 10 and 16 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable

over claims 1, 2, 5, 10, and 23 of copending Application No. 10/703,906. Claims 1, 6, 10, and 16 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 18 of copending Application No. 11/053,551. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Enclosed with this response is a Terminal Disclaimer to obviate the double patenting rejection over U.S. Patent No. 6,593,116, U.S. Patent No. 6,913,911, U.S. Application No. 10/703,906, and U.S. Application No. 11/053,551 along with five statements under 37 C.F.R. § 3.73(b).

Claim Objections

The Examiner has objected to claim 15. Applicants respectfully traverse this objection to the extent that it is applied to the claims as amended.

Claim 15 has been cancelled, therefore, the rejection is moot.

Allowance of claims 1, 6, 7, 10, and 14-21, is respectfully solicited.

Respectfully submitted,

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Table 2. DEVELOPMENT OF ACTIVE SENSITIZATION TO TUBERCULIN IN GUINEA PIGS RECEIVING DISRUPTED CELLS FROM PERIPHERAL BLOOD, SPLEEN, AND LYMPH NODES OF HYPERSENSITIVE DONORS

		Days after injection of cells						
		0	7	13–14	17–25	33–43	61–65	82–85
	Peripheral leucocytes (~1 × 10⁸ cells)							
(a)	No. tested	3	6	8	5	11	8	8
	Strong reactions*	0	0	0	2	4	4	3
	Weak reactions	0	1	4	1	4	1	1
	Anaphylaxis†							7 (3 died)
	Spleen (~5 × 10⁸ cells)							
(b)	No. tested	3	6	9	6	12	9	9
	Strong reactions	0	0	0	0	0	2	1
	Weak reactions	0	1	4	3	7	1	3
	Anaphylaxis							8 (6 died)
	Lymph node cells (~6 × 10⁸ cells)							
(c)	No. tested	—	—	9	3	6	3	3
	Strong reactions	—	—	0	1	1	1	1
	Weak reactions	—	—	4	0	2	0	1
	Anaphylaxis							2 (1 died)
	No cells							
(d)	No. tested	9	9	—	9	—	9	9
	Strong reactions	0	0	—	0	—	0	0
	Weak reactions	0	1	—	0	—	0	2
	Anaphylaxis							3 (none died)

* Strong reactions were >10 × 10 mm.; weak reactions 7 × 7 to 10 × 10 mm. † Tested with 2 mgm. *PPD* intravenous.

stage or other and only 2 out of the 18 animals, which were followed over the whole period, failed to show any reaction. As a control, 9 normal animals were skin tested with *PPD* 5 times over a similar period. One of these showed reactivity on the second injection 7 days after the first, and afterwards failed to react, and 2 others showed very mild reactions which appeared only at the end of the three months course. When frozen and thawed peripheral blood leucocytes and spleen cells from normal unsensitized animals were used the recipients behaved like the control group with no cells.

At the end of the experiment all four groups were challenged with 2 mgm. *PPD* intravenously. Sixteen out of 18 animals which had received disrupted cells from hypersensitive donors developed anaphylaxis and 10 died. Only 2 of the 9 controls developed very mild anaphylaxis.

From these results it appears that a transient delayed hypersensitivity is transferred by intact peripheral blood leucocytes, spleen cells and lymph node cells, and that it may be followed by a superimposed active immunization which persists far beyond the time when the donor cells would have been destroyed by a homograft reaction. Peripheral blood leucocytes were more effective in respect of active sensitization than cells from the spleen and lymph nodes. A further property of cells from sensitized donors is that they can enhance the development of antibody against tuberculin accompanying multiple injections of *PPD*. *PPD* is not a single substance, and there is no evidence whether the antibody is directed against traces of contaminating antigen which are known to be present.

These experiments indicate that the possibility that active immunization has occurred must be borne in mind in the interpretation of experiments on passive transfer of delayed hypersensitivity.

I would like to thank Dr. J. H. Humphrey for his advice throughout this investigation.

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ENZYMATIC SYNTHESIS OF POLY-β-HYDROXYBUTYRIC ACID IN BACTERIA

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THE polymeric ester, poly-β-hydroxybutyric acid, originally discovered by Lemoigne in 1927 as a major component of *Bacillus megaterium*, has since been found to be an important storage material in a variety of bacteria[1–4]. It serves as an intracellular reserve of carbon and reducing power in the photosynthetic bacterium, *Rhodospirillum rubrum*, and as the principal substrate for the endogenous respiration of certain aerobes[3–5].

The present report deals with the synthesis of the polymer from carbon-14-labelled D(–)-β-hydroxybutyryl-coenzyme *A* by particulate fractions of cell-free extracts from *B. megaterium* and *R. rubrum*.

Radioactive D(–)-β-hydroxybutyric acid was obtained by alkaline hydrolysis of purified polymer isolated from cells of *R. rubrum* that had assimilated uniformly labelled acetate in the light[1]. The acid was purified by paper chromatography, and the coenzyme *A* derivative was prepared by the mixed anhydride method of Wieland and Rueff[6]. Free β-hydroxybutyric acid was removed from the acidified solution by ether extraction. Analysis of the specific

Table 1. SYNTHESIS OF POLYMER BY *B. megaterium* EXTRACTS

Time (min.)	Percentage carbon-14 recovered in polymer fraction Exp. 1	Exp. 2	Exp. 3
0	0	0	0
5	—	27	—
10	—	35	—
20	33	45	46
40	36	44	—

The reaction mixtures contained either recombined particulate and supernatant fractions (1·08 mgm. polymer and 1·75 mgm. protein per ml.) or resuspended particulate fraction alone (1·08 mgm. polymer and 0·13 mgm. protein per ml.); and the following components (in μmoles per ml.): magnesium chloride, 12·5; glutathione, 12·5; and potassium phosphate buffer, pH 7·4, 125.

Exp. 1: particulate + supernatant fractions + 0·48 μequiv. D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial); Exps. 2 and 3: particulate fraction + 0·24 and 0·48 μequiv. D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial), respectively.

The reaction mixtures were incubated at 33° C.

activity of the product, coupled with hydroxamate and sulphydryl determinations, indicated that at least 95 per cent of the acid was present in thioester combination. Since the commercially available coenzyme *A* used for the synthesis is only 50–75 per cent pure and is contaminated with other sulphydryl compounds (for example, glutathione), the assays reflect the total concentration of β-hydroxybutyryl thioesters, rather than of the coenzyme *A* derivative alone. Poly-β-hydroxybutyric acid was assayed with a modified hydroxamate method for ester determination[7], or by the spectrophotometric method of Slepecky and Law[8].

B. megaterium, strain Km, was grown in the medium containing 2 per cent glucose described by Macrae and Wilkinson[4]. The cells were gathered at the end of exponential growth and washed with 0·033 *M* phosphate buffer, pH 7·0. Cell-free extracts were prepared by the osmotic lysis of concentrated protoplasts made by lysozyme treatment of the cells[9]. The lysates were subjected to sonic oscillation to liberate the polymer particles from the cell membranes. The heavy particulate fraction (consisting mainly of polymer particles) was separated from the extract by centrifugation at 3,000 *g* for 20 min. at 4° C., and the particles were washed several times with phosphate buffer, pH 7·0. The supernatant fraction contained both the soluble proteins of the cell and the disintegrated cell membranes.

The particulate fraction was incubated with radioactive β-hydroxybutyryl-coenzyme *A* in the presence and absence of the supernatant fraction. The reaction was stopped by addition of alcohol (60 per cent final concentration) and the radioactivity of the isolated polymer was measured. The particles were sedimented by centrifugation, treated with alkaline hypochlorite, washed successively with water and acetone and finally dissolved in chloroform[3,4]. Suitable aliquots were analysed for radioactivity. Controls were performed with reaction mixtures in which β-hydroxybutyryl-coenzyme *A* was replaced by labelled β-hydroxybutyrate in the presence or absence of coenzyme *A*, as well as by alkali-hydrolysed coenzyme *A* derivative. No incorporation of radioactivity into polymer was observed in the control reaction mixtures. In the presence of β-hydroxybutyryl-coenzyme *A*, on the other hand, the polymer became rapidly labelled with carbon-14 when the particulate fraction was used either alone or in combination with the supernatant fraction (Table 1).

The reaction came to completion in about 20 min., at which time 35–45 per cent of the total radioactivity was recovered in the polymer. The observed maximal conversion of thioester to polymer does not give any information on the equilibrium constant for the synthesis of polymer from β-hydroxybutyryl-coenzyme *A*, since the true concentration of the latter compound was not known for reasons previously discussed.

To study the stoichiometry of the reaction, a further experiment was performed in which the disappearance of thioester was measured. The analyses showed that in a reaction mixture initially containing 1·2 mgm. washed polymer particles (without supernatant fraction) and 1·2 μequiv. of thioester per ml., 0·5 μequiv. of β-hydroxybutyrate was incorporated in the polymer, while 0·6 μequiv. of thioester had disappeared. Thus, the synthesis of polymer accounted for more than 80 per cent of the thioester decomposed.

To identify the product unequivocally, the labelled polymer from the above experiment was diluted with purified carrier polymer[3,4] and reprecipitated three times from chloroform: twice with 95 per cent ethanol and once with acetone–ether (2 : 1). The specific activity remained constant throughout. The reprecipitated polymer was hydrolysed for 22 hr. at 30° in *N* potassium hydroxide, treated with cation exchange resin ('Dowex 50', H⁺), and chromatographed on paper using the following solvents: (1) ether–benzene–formic acid–water (50 : 50 : 12·5 : 2); (2) *n*-butanol–glacial acetic acid–water (100 : 6 : 25); (3) *n*-butanol saturated with 1·5 *M* ammonia. In each case, only one radioactive spot was found on the paper, which corresponded exactly with the location of the β-hydroxybutyric acid spot.

Cell-free extracts of *R. rubrum* also rapidly incorporate the β-hydroxybutyryl moiety of the coenzyme *A* derivative into polymer. As in *B. megaterium*, the activity is largely associated with the particulate fraction. The kinetics of incorporation are, however, complicated by the presence of an active depolymerase which is also associated with the particles. Crude extracts were prepared by sonic oscillation of cells grown anaerobically in the light. The particulate and supernatant fractions were separated by low-speed centrifuging. The former, which contained the polymer particles, was heavily contaminated with pigmented cell debris. The native polymer, when resuspended in either the supernatant fraction or buffer, underwent rapid hydrolysis to β-hydroxybutyric acid. As much as 80 per cent of the polymer disappeared after 30 min. of incubation at 33° C.

The results of an experiment in which labelled β-hydroxybutyryl-coenzyme *A* was added to a crude cell-free preparation are shown in Fig. 1. Maximal incorporation was achieved within 2 min., after which time the total carbon-14 content of the polymer rapidly decreased. The early termination of incorporation can be plausibly ascribed to exhaustion of the substrate. Thereafter, radioactivity is lost from the polymer as a consequence of the continued depolymerization. The results of a parallel experiment, in which the resuspended particulate fraction was incubated with different concentrations of substrate, are entirely compatible with this interpretation (Table 2). The identification of the carbon-14-labelled product was carried out as described above in the experiments with *B. megaterium*.

No direct activation of β-hydroxybutyric acid with adenosine triphosphate and coenzyme *A* could be demonstrated in cell-free extracts of *R. rubrum*. A small incorporation of carbon-14 into polymer was

VOL. 189 ... ould have Peripheral respect of spleen and cells from hance the lin accom- D is not a e whether f contami- sent. possibility must be xperiments tivity. rey for his ... ID ... ms of cell- rubrum. d was ob- d polymer assimilated The acid , and the the mixed off. Free he acidified the specific

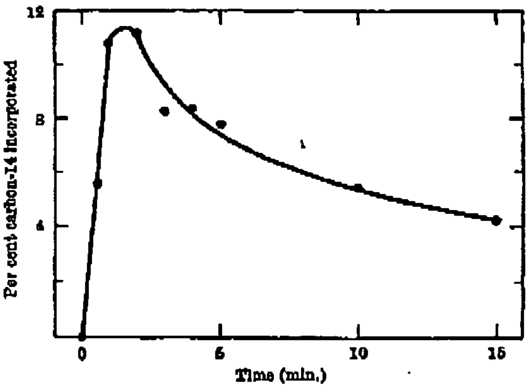

Fig. 1. Incorporation of carbon-14 into polymer by crude extracts of *R. rubrum*. The reaction mixtures contained crude extract (3·9 mgm. polymer and 5·03 mgm. protein per ml.); D(−)-β-hydroxybutyryl-coenzyme *A*, 1·4 μequiv.; and the following components (in μmoles per ml.): magnesium chloride, 12·7; glutathione, 12·7; and *tris* (hydroxymethyl) aminomethanehydrochloride buffer, pH 7·4, 127. The reaction mixtures were incubated at 33° C.

observed, however, when crude extracts were incubated with carbon-14-labelled acetate, adenosine triphosphate, coenzyme *A* and reduced pyridine nucleotides.

Two soluble enzymes, presumably involved in polymer metabolism, were found in the supernatant fraction of *R. rubrum* extracts prepared from cells that had depleted their polymer stores. A hydrolytic system catalyses digestion of boiled polymer particles of *R. rubrum* and native polymer particles of *B. megaterium* but does not attack the purified polymer. A specific diphosphopyridine nucleotide-linked D(−)-β-hydroxybutyric acid dehydrogenase is also present in such extracts, and has been partially purified.

Table 2. SYNTHESIS OF POLYMER BY PARTICULATE FRACTION OF *R. rubrum* EXTRACTS

Time (min.)	Percentage carbon-14 recovered in polymer fraction Exp. 1	Exp. 2	Exp. 3
2	9	10	7
5	6	9	8
10	5	7	11

The reaction mixtures contained resuspended particulate fraction (3·1 mgm. polymer per ml.); and the following components (in μmoles per ml.): magnesium chloride, 10; glutathione, 10; and *tris* (hydroxymethyl) aminomethanehydrochloride buffer, pH 7·4, 100. Exps. 1, 2 and 3 contained 0·25, 1·0 and 4·0 μequiv. of D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial), respectively. The reaction mixtures were incubated at 33° C.

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INHIBITION OF CELLULAR RESPIRATION BY CO-CARCINOGENIC FRACTIONS OF CROTON OIL

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As a result of the work of Rous and his colleagues[1] and Berenblum and his colleagues[2], it is widely accepted that carcinogenesis in skin—and probably also in other organs—takes place in two successive stages. A single small dose of a carcinogen such as 9 : 10-dimethyl-1 : 2-benzanthracene applied to mouse skin is sufficient to produce a lasting 'initiation' but no tumours. Subsequent repeated application of croton oil gives rise to a large crop of tumours, whereas croton oil applied without a previous initiating stimulus produces few, if any, tumours. Several arguments[3] make it clear that the 'promotion' or 'co-carcinogenesis' by croton oil is complementary to initiation and quite distinct from it; thus, administration of the initiator after prolonged treatment with croton oil does not lead to tumour formation.

The factor or factors in croton oil responsible for tumour promotion have not been identified, and their effects on cell metabolism have not previously been described. Lijinsky[4] has submitted methanolic extracts of croton seeds to silica-gel chromatography and obtained fractions, comprising about 1 per cent of the whole oil, that appear to have all its tumour-promoting activity. Fractions kindly provided by Dr. Lijinsky are at present being analysed by gas-chromatography in this Institute in attempts to identify the active agent or agents. In the meantime, the opportunity has been taken—for reasons which will be apparent in the discussion here—to analyse the effects of the croton oil fractions on cell metabolism. It has been found that the highly active tumour-promoting fractions are inhibitors of cellular respiration in several tissues, and this property may well be related to their co-carcinogenic effect.

Rat liver homogenates. Livers of 100-gm. hooded rats were homogenized by the Potter–Elvehjem technique in the following medium: 0·3 *M* sucrose, 0·002 *M* sodium ethylenediamine tetraacetate and 0·03 *M* nicotinamide, adjusted to pH 7·1 with potassium hydroxide. Each Warburg flask contained 0·3 ml. homogenate (containing 1·02 mgm. protein nitrogen per ml.), 0·2 ml. potassium succinate, 0·15 *M*, pH 7·1 and 1·4 ml. sucrose–ethylenediamine tetraacetate–nicotinamide as above. Croton oil fractions were added in ten-fold dilutions in 0·05 ml. ethanolic solution to give final concentrations from 0·2 to 200 μgm. per ml. Oxygen uptake from air